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Exploring the anti-cancer potential of *Ixora* extracts: A multi-cell line approach

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ABSTRACT: Bioactive anticancer compounds like alkaloid camptothecin and flavonoids rutin, quercetin and kaempferol have been isolated from *Ixora coccinea*. Being commercially important, research towards finding more plants with anticancer compounds belonging to genus *Ixora* is on the forefront. Anti cell proliferative potential of ethanolic plant part extracts of *I. pavetta, I. barbata, I. javanica and I. undulata* were studied against three cancer cell lines namely, Human leukemia cancer cell line K-562, Human breast cancer cell line MDA-MB-231 and Human lung cancer cell line A-549 using SRB assay. The result of anti-cell proliferative activity is further correlated with the total flavonoid content of aerial parts and total alkaloid content of underground parts of plants estimated by spectrophotometric assay. The root extract of *I. javanica* exhibited the best results with GI_{50} values of <10 µg/ml against all the three cancer cell lines studied. Interestingly, the total alkaloid content was also highest in roots of *I. javanica* possibly pointing some novel alkaloid to be responsible for its strong anti-cell proliferative activity. Bark extracts of *I. javanica, I. pavetta* and *I. barbata* also exhibited GI_{50} values of <10 µg/ml specifically against one of the cell lines studied namely - Human leukemia cancer cell line K-562. All three barks mentioned above also showed higher levels of total alkaloids. This is the first study reporting the anti-cell proliferative potential of root and bark extracts of *I. javanica* and *I. barbata*. These plants possess in their roots and bark some alkaloids with strong anti-cell proliferative activity which needs further investigation.

KEYWORDS: *Ixora* species; flavonoid content; alkaloid content; K-562 cell line; MDA-MB-231 cell line; A-549 cell line; anti-cell proliferative; SRB assay.

1. INTRODUCTION

Genus *Ixora* belongs to the family Rubiaceae and is characterized by the presence of bioactive metabolites with great pharmacological potential. Plant parts of several *Ixora* species are used in the Indian traditional system of medicine. *Ixora coccinea* has been well investigated and shown to exhibit antinociceptive, hepatoprotective [1], antimicrobial [2] cytotoxic, anticancer [3] and anti-inflammatory properties [4].

Secondary metabolites isolated from plant sources belonging to the phenolic group have long been known to exhibit anti cell proliferative activity [5]. Phytochemical investigation of *Ixora coccinea* points to the presence of several phenolic compounds belonging to flavonoid class, such as rutin, quercetin and kaempferol in the leaves and flowers of the plant [6]. Rutin has been reported to have anti-cell proliferative effects on several cell lines including human leukemia HL-60 cell line [7] and human colon cancer SW-480 cell line [8]. Kaempferol is considered to have anti-cancer potential as it exerts cytotoxic effects in many types of cancer cells [9]. Quercetin is reported to result in apoptosis by downregulating the expression of various oncogenes [10].

Alkaloids are yet another major class of nitrogen containing secondary metabolites known to have anticancer potential. Camptothecin from the bark of *Camptotheca acuminata* is an important alkaloid known for its anticancer properties [11]. Camptothecin has also been reported in the roots and barks of *Chonemorpha fragrans* [12] and *Ixora coccinea* [6,13,14]. Research towards finding more plants with this rare alkaloid camptothecin is on the forefront. Presence of novel drugs with comparable properties are often screened from allied species. In our preliminary study we found that the bark and roots of some *Ixora* species under study showed high alkaloid content and the flowers and leaves of these *Ixora* species showed high flavonoid content. *Ixora barbata* bark and root was evaluated for its camptothecin content by high performance thin layer chromatography (HPTLC) method and compared with *Ixora coccinea* [15]. It was observed that bark of *Ixora*

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barbata had higher alkaloid content than *I. coccinea* and can be used as the target plant for commercially obtaining this important drug. Amongst species of *Ixora* widely used for medicinal purposes, very few reports are available on the anti-cancer properties of some plant parts of *Ixora javanica* [16, 17] and *Ixora undulata* [18,19]. Additionally, there are no reports available on the anti-cell proliferative or anti-cancer properties of *Ixora pavetta* and *Ixora barbata*. The current study aimed at investigating the anti-cell proliferative activity of ethanolic extracts of various plant parts of the four above mentioned *Ixora* species against three selected cancer cell lines using SRB assay. These results are further correlated with total alkaloid content in the bark and root extracts and total flavonoid content in the flower and leaf extracts of 4 *Ixora* species under study. Using this study as the base data, the metabolite profiling for selected plant parts may also assist in finding the phytochemical possibly responsible for anticancer activity. This study will thus be an excellent material for any researcher to start looking for novel anti-cell proliferative metabolites with possibly strong anticancer activity in selectively suggested *Ixora* species (or plant parts).

2. RESULTS

2.1. Studies on anti-cell proliferative activity

2.1.1. Cell proliferation inhibitory activity exhibited by standard flavonoids rutin, quercetin and kaempferol and standard alkaloid camptothecin

Flavonoid rutin did not show inhibition of cell proliferation in any of the cell lines used in this study. Flavonoid quercetin showed GI_{50} values of less than <10 µg/ml against 2 of the 3 cell lines studied namely, A-549 and K-562. Further, quercetin showed a GI_{50} value of 42 µg/ml against MDA-MB -231 cell line indicating mild anti cell proliferative activity. Flavonoid kaempferol (Std- 3) showed GI_{50} values of less than <10 µg/ml against K-562 cell line and GI_{50} values of 68.5 µg/ml against A-549 cell line but no inhibition of cell proliferation was noted against MDA-MB-231 cell line. Alkaloid camptothecin (Std- 4) and positive control Adriamycin showed GI_{50} values of less than <10 µg/ml against all the three cell lines under study. (Table 1 and 2, Figure 1).

Table 1 Effect of plant extracts and standard drugs on cancer cell lines represented as % control growth.

	Human Leukemia Cancer Cell Line K-562				Human Lung Cancer Cell Line A-549				Human Breast Cancer Cell Line MDA-MB-231			
	% Control Growth Drug Concentrations (µg/ml) Average Values				% Control Growth Drug Concentrations (µg/ml) Average Values				% Control Growth Drug Concentrations (µg/ml) Average Values			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**
STD-1	69.5	76.7	77.9	93.9	93.5	96.0	90.9	94.5	80.0	84.0	92.7	82.3
STD-2	22.0	1.2	13.4	23.1	40.2	29.1	14.2	9.7	60.5	44.8	47.8	48.5
STD-3	48.4	32.9	29.4	28.5	90.2	79.3	56.7	47.4	89.0	86.7	67.8	64.2
STD-4	10.2	6.7	13.6	15.4	5.0	7.3	-2.4	8.3	2.9	8.9	11.1	5.5
ADR	-6.7	-4.1	5.6	-9.9	7.0	5.8	4.0	-4.3	-70.1	-65.2	-64.7	-59.2
IJRT	3.4	15.6	2.5	7.9	51.1	23.2	-30.8	-60.6	70.0	-32.5	-60.3	-51.4
IJBK	43.4	54.0	20.9	49.8	113.6	114.9	102.4	77.8	77.3	82.7	85.8	62.4
IBBK	46.7	59.5	37.1	46.5	105.0	100.4	93.1	74.1	75.7	77.5	79.6	68.3
IPBK	49.1	51.0	62.3	59.1	130.0	129.6	131.5	136.5	74.5	84.4	82.4	80.7
IBLF	29.1	22.3	46.0	74.9	85.4	91.4	92.3	85.5	87.1	87.6	74.0	69.1
IBFL	20.8	28.8	41.3	46.0	120.1	132.9	133.4	114.5	71.0	68.2	66.0	44.6
IJLF	27.7	39.1	48.7	71.7	98.7	104.9	107.3	113.5	82.4	83.7	71.8	65.0
IJFL	22.2	24.1	46.8	54.5	126.5	136.2	135.8	142.8	74.6	74.1	69.7	64.3
IPLF	34.3	48.5	56.9	96.8	108.5	122.9	129.3	126.6	90.0	93.3	84.4	85.8

IJRT- *I. javanica* root; IJBK- *I. javanica* bark; IBBK- *Ixora barbata* bark; IPBK- *Ixora pavetta* bark; IBLF- *Ixora barbata* leaf; IBFL- *Ixora barbata* flower; IJLF- *Ixora javanica* leaf; IJFL- *Ixora javanica* flower; IPLF- *Ixora pavetta* leaf. (Std-1) - Rutin, (Std-2) - Quercetin, (Std-3) - Kaempferol, (Std-4) - Camptothecin, ADR- Adriamycin, Positive control compound.

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Table 2. Effect of plant extracts and standard drugs on cancer cell lines represented as LC50, TGI and GI50 values.

	Drug concentrations (µg/ml) calculated from graph								
	Human Leukemia Cancer Cell Line K-562			Human Lung Cancer Cell Line A-549			Human Breast Cancer Cell Line MDA-MB-231		
	LC50	TGI	GI50*	LC50	TGI	GI50*	LC50	TGI	GI50*
STD-1	NE	NE	>80	NE	NE	>80	NE	NE	>80
STD-2	NE	NE	<10	NE	NE	<10	NE	NE	**42**
STD-3	NE	NE	<10	NE	NE	**68.5**	NE	NE	>80
STD-4	NE	NE	<10	NE	NE	<10	NE	NE	<10
ADR	NE	NE	<10	NE	NE	<10	NE	NE	<10
IJRT	NE	NE	<10	**66.9**	**34.7**	<10	**62.0**	**23.0**	<10
IJBK	NE	NE	<10	NE	NE	>80	NE	NE	>80
IBBK	NE	NE	<10	NE	NE	>80	NE	NE	>80
IPBK	NE	NE	<10	NE	NE	>80	NE	NE	>80
IBLF	NE	NE	**46.9**	NE	NE	>80	NE	NE	>80
IBFL	NE	NE	>80	NE	NE	>80	NE	NE	**70.4**
IJLF	NE	NE	**42.8**	NE	NE	>80	NE	NE	>80
IJFL	NE	NE	**64.4**	NE	NE	>80	NE	NE	>80
IPLF	NE	NE	**26.8**	NE	NE	>80	NE	NE	>80

LC50 = Concentration of drug causing 50% cell kill. GI50 = Concentration of drug causing 50% inhibition of cell growth. TGI = Concentration of drug causing total inhibition of cell growth. ADR = Adriamycin, Positive control compound. NE-Non evaluable data. GI50* value of ≤ 10^-6 molar (i.e., 1 µmolar) or ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 20µg/ml is considered to demonstrate anti cell proliferative activity. Yellow highlighted are test values with GI50<10.

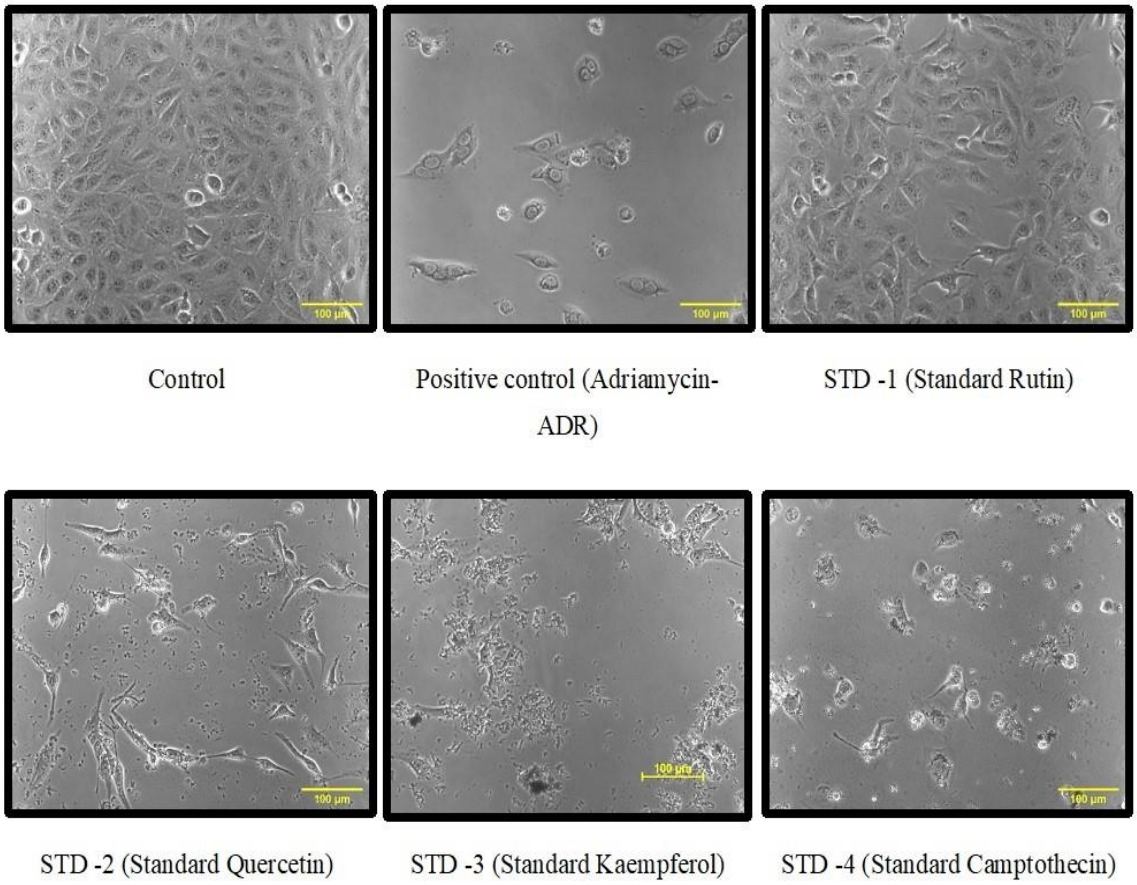

Figure 1. Images showing effect of control sample, Adriamycin (positive control), STD-1, STD-2, STD-3, and STD-4 against all three cell lines. Cells observed under 20 X magnification.

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2.1.2. Root and Bark samples with cell proliferation inhibitory activity

Root extract of *I. javanica* exhibited the best anti-cell proliferative activity with GI_{50} values of <10 µg/ml for all the three cancer cell lines evaluated (Table 1 and 2, Figures 2, 3 and 4). It also showed an LC_{50} value of 66.9 µg/ml and TGI value of 34.7 µg/ml against A-549 cell line (Table 1 and 2, Figure 2).

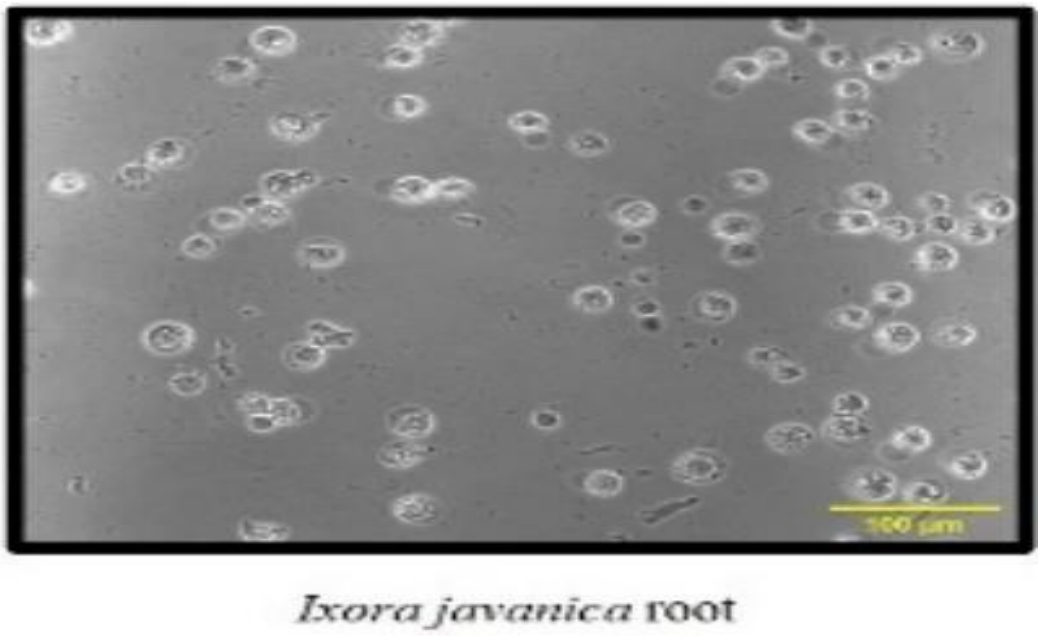

Figure 2. Image showing strong inhibition of cell proliferation effected by ethanolic root extract of *Ixora javanica* against A-549 cancer cell line. Cells observed under 20 X magnification.

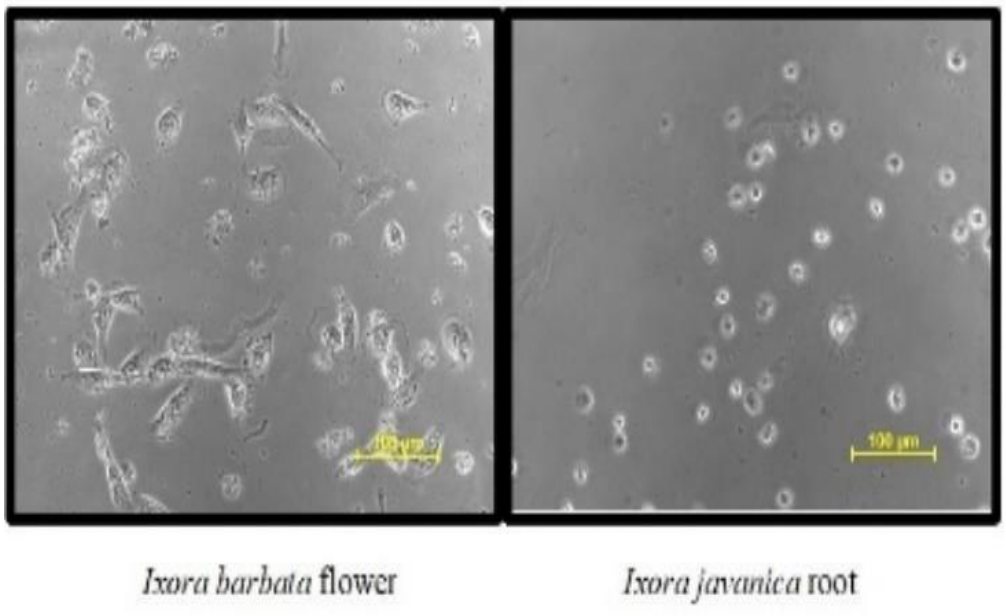

Figure 3. Images of *Ixora barbata* ethanolic flower extract showing mild anti-cell proliferative activity against MDA-MB-231 cancer cell line and *Ixora javanica* ethanolic root extract showing strong inhibitory activity against MDA-MB-231 cancer cell line. Cells observed under 20 X magnification.

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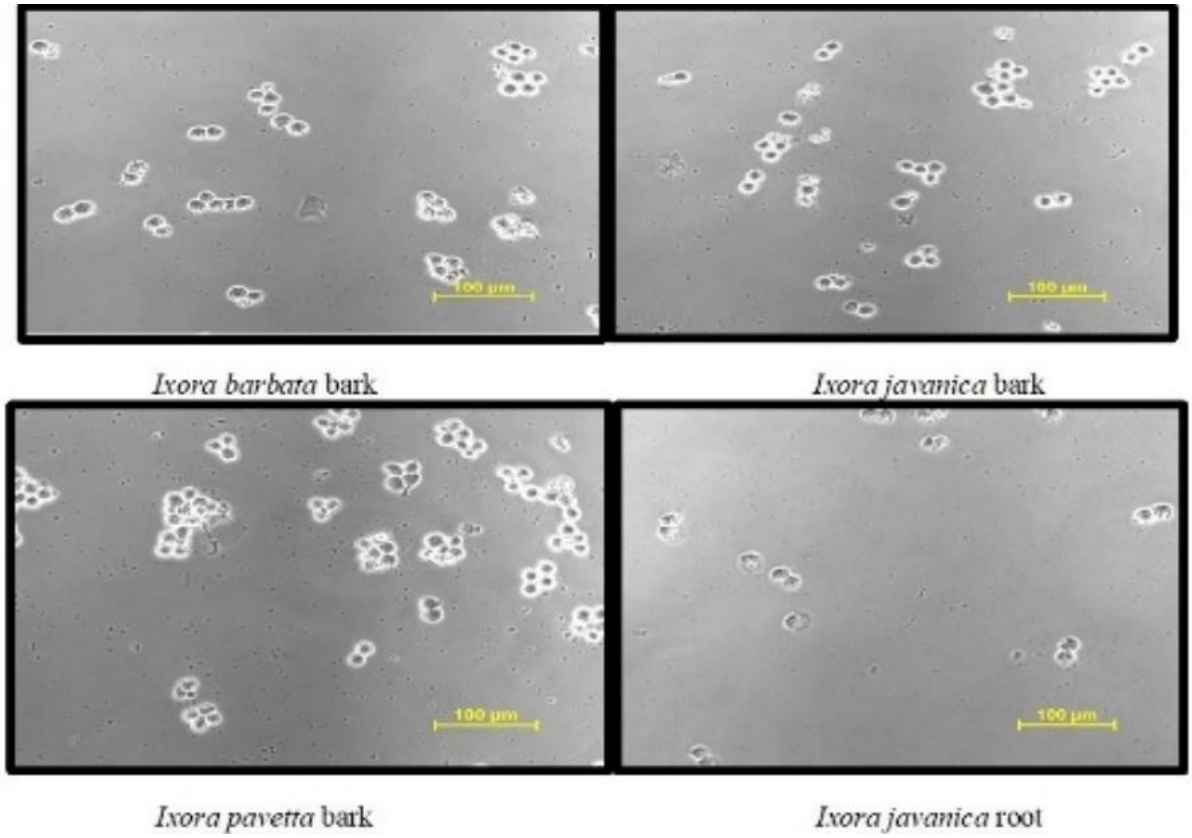

Figure 4. Images of ethanolic bark extracts of *Ixora barbata, Ixora javanica, Ixora pavetta* and ethanolic root extracts of *Ixora javanica* showing strong anti-cell proliferation activity against K-562 cancer cell line. Cells observed under 20 X magnification.

Bark extracts of *I. javanica, I. pavetta* and *I. barbata* also exhibited GI_{50} values of <10 µg/ml specifically against K-562 cell line (Table 1 and 2, Figure 4). These extracts however did not show any inhibitory activity against the A-549 and MDA-MB-231 cell lines (Table 1 and 2). The bark extracts of *I. undulata* did not show any anti cell proliferative activity against any of the three cell lines under study.

2.1.3. Leaf and flower samples with mild anti-cell proliferation activity

Among the leaf and flower samples studied GI_{50} values of <10 µg/ml were not observed in any of the samples studied (Table 2). Ethanolic leaf and flower extracts of *I. javanica* gave GI_{50} value of 42.8 µg/ml and 64.4 µg/ml respectively against K-562 indicating mild anti-cell proliferative activity towards this cell line (Table 2 and Figure 5). A lower GI_{50} value of 26.8 was observed in only leaf ethanolic extracts of *I. pavetta* against K-562 cell line (Table 2 and Figure 5). Ethanolic leaf extract of *I. barbata* showed a GI_{50} value of 46.9 µg/ml against K-562 cell line (Table 2 and Figure 5). The ethanolic flower extracts of *I. barbata* also showed GI_{50} value of 70.4 µg/ml against MDA-MB-231 cell line indicating mild anti-cell proliferative activity (Table 2 and Figure 3).

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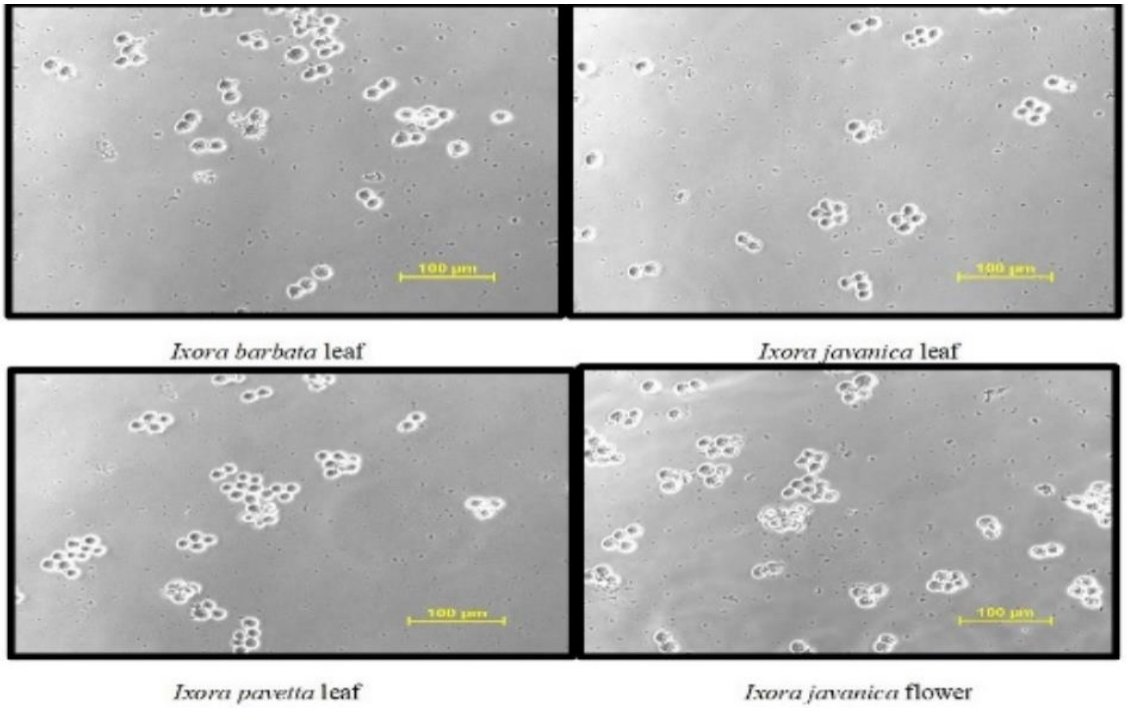

Figure 5. Images of ethanolic leaf extracts of *Ixora barbata, Ixora javanica*, *Ixora pavetta* and flower extracts of *Ixora javanica* showing mild cell proliferation inhibitory activity against K-562 cancer cell line. Cells observed under 20 X magnification.

2.1.4. Alkaloid content of root and bark samples

The alkaloid content of all bark and root extracts are reported in Table 3. The root extract of *I. javanica* had the highest total alkaloid content. Although the total alkaloid content in the ethanolic bark extracts of *I. javanica, I. pavetta* and *I. barbata,* were also appreciably high, they were lesser than the ethanolic root extracts of *I. javanica* which showed the best anti-cell proliferation activity.

Table 3. Comparative account of total alkaloid in root, bark samples and total flavonoid content in leaf and flower samples of various *Ixora* species.

***Ixora* species and parts (ethanolic extract)**	**Total Alkaloid content (mg/gm)**	**Total Flavonoid content (mg/gm)**
***I. javanica* Root**	**106 ±1.22**	-
***I. barbata* Root**	96 ±1.13	-
***I. javanica* Bark**	84 ±1.24	-
***I. barbata* Bark**	88 ±1.08	-
***I. pavetta* Bark**	100 ±1.31	-
I. undulata Bark	82 ±1.24	-
***I. javanica* Flower**	-	**230.7 ±1.38**
***I. barbata* Flower**	-	84.6 ±1.03
***I. javanica* Leaf**	-	219.2 ±0.89
***I. barbata* Leaf**	-	184.6 ±1.12
***I. pavetta* Leaf**	-	207.6 ±0.99
***I. undulata* Leaf**	-	226.9 ±1.08

The data represents mean values of 3 replicates ± SE at 95% confidence with ($p < 0.05$).

2.1.5. Flavonoid content of leaf and flower samples

The flavonoid content of all leaf and flower extracts are reported in Table 3. A remarkably higher total flavonoid content was observed in the ethanolic extracts of flowers (230.7 mg/gm) and leaves (219.2 mg/gm)

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of I. *javanica*, and leaf extracts (207.6 mg/gm) of *I. pavetta.* This was followed further by leaf extracts (184.6 mg/gm) and flower extracts (84.6 mg/gm) of *I. barbata.*

3. DISCUSSION

Flavonoids are usually known to exert anticancer effects. Rutin has been reported to have anticancer effects on several cell lines such as neuroblastoma LAN-5 cell line [20], colorectal CRC cell line [21], pulmonary metastasis B16F10 cell line [22], melanotic melanoma B16 cell line [23] and hepatic carcinoma HTC cell line [24]. However, flavonoid rutin did not show anti-cell proliferative activity in any of the cell lines used in this study. Flavonoid quercetin showed strong inhibition against A-549 and K-562 cell lines and mild inhibition against MDA-MB -231 cell line. Anti cell proliferative activity of quercetin against different cancer cell lines has also been reported earlier [25,26]. As kaempferol showed strong inhibitory activity against K-562 cell line, mild inhibitory activity against A-549 cell line and no activity against MDA-MB -231 cell line, it points to the fact that flavonoids quercetin and kaempferol have anti-cell proliferative activity only towards selective cell lines.

Alkaloid camptothecin showed strong anti-cell proliferative activity against all the three cell lines under study. This proves the strong anticancer potential of alkaloid camptothecin against multiple cell lines. There is a current need to screen plants containing a higher amount of alkaloids showing strong anti-cell proliferative activity and further screen those for the presence of alkaloid camptothecin. Among all the samples analyzed in this study, the root extract of *I. javanica* exhibited strongest inhibitory activity against all the three cancer cell lines studied. Its inhibitory activity was comparable to positive control adriamycin. Additionally, amongst all the root and bark samples studied, root extract of *I. javanica* was also detected with the highest total alkaloid content. A strong anti-cell proliferative activity against all three cell lines exhibited by *I. javanica* root ethanolic extracts along with a high alkaloid content makes the root of this plant the best target to search for novel anticancer compounds. *I. barbata* root, although showed higher alkaloid content, did not show anti-cell proliferative activity against any of the cell lines under the present study. However, on account of its high alkaloid content, *I. barbata* root should also be investigated further for inhibitory activities against other cell lines. The total alkaloid content in the ethanolic bark extracts of *I. javanica, I. pavetta* and *I. barbata,* although appreciably high, was a little lesser than that reported in the ethanolic root extracts of *I. javanica.* Alkaloid, camptothecin present in *I.coccinea* is reported to possess anti-cell proliferative property and exerts cytotoxic effects in many types of cancer cells [13,27,28,29]. Camptothecin content in the four species of *Ixora* also needs to be investigated to establish its relationship, if any, with the presently observed anti-cell proliferative activity in both bark and root samples.

Among the leaf and flower samples studied GI_{50} values of less than 10 µg/ml were not observed in any sample. However, anti-cell proliferative activity in the closer range of GI_{50} value <80 µg/ml was detected in some of the flower and leaf samples of *Ixora*. A higher total flavonoid content observed in the ethanolic flower and leaf extracts of *I.javanica* accompanied a mild anti-cell proliferative activity against one of the cell lines (K-562) studied. In an earlier study using HPTLC analysis, the highest amount of flavonoid kaempferol (527.8 µg/ml) was reported in the ethanolic leaf extracts of *I. javanica.* The same study also reported presence of kaempferol (92.8 µg/ml) in flower extracts of the same plant [30]. Kaempferol is considered to have anti-cancer potential and exerts cytotoxic effects in many types of cancer cells [9]. This may point to the fact that a high flavonoid amount with high kaempferol content may be responsible for mild anti-cell proliferative activity seen in leaf and flower extracts of *I. javanica* especially against (K-562) cell line studied. Ethanolic leaf extract of *I.barbata* with higher flavonoid content of 184.6 mg/gm also showed a mild anti-cell proliferative activity (GI_{50}=46.9) against (K-562) cancer cell line. The remarkably higher flavonoid content in *I. pavetta* (207.6 mg/gm) was accompanied by its comparatively stronger inhibitory potential (GI_{50}=26.8) against the K-562 cell line. As standard flavonoid kaempferol also showed strong anti-cell proliferative activity especially towards the K-562 cell line, the amount of flavonoid kaempferol in *I. pavetta* could be investigated further. Thus, some specific flavonoids or even flavonoid kaempferol may be responsible for this mild anti-cell proliferative activity in *I. barbata*, *I. javanica and I. pavetta.* However, leaf of *I. undulata* proved to be an exception as it did not show any anti-cell proliferative activity despite comparatively higher quantity of total flavonoids. Possibly *I. undulata* contains flavonoids other than the ones responsible for anticancer activity or may be active against other cell lines not investigated in this study. The rest of the flower and leaf samples showed GI_{50} >80µg/ml and therefore were not considered to show any anti-cell proliferation activity against any of the cell lines under this study.

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4. CONCLUSION

From the above result it is concluded that all parts of *I. javanica* are of great pharmacological value and need to be studied further to assess its strong and potent inhibitory activity towards cell proliferation against all three cancer lines. *I. barbata* is the second in line to be investigated for identification and isolation of pharmacologically important anticancer alkaloids and flavonoids. Synergistic activity of multiple metabolites may also be responsible for strong anti-cell proliferative activity and mechanism underlying the same need to be further studied. Additionally, *I. pavetta* was studied with respect to only leaf and bark samples as roots and flowers were unavailable for its study. The bark extracts of *I. pavetta* also showed a GI_{50} value of <10 and the leaf extracts showed GI_{50} value of < 80 µg/ml against K-562 cell line. Total flavonoid content in the leaf extracts of *I. pavetta* and the total alkaloid content in its bark sample were remarkably high. *I. pavetta* therefore is third in line to be further investigated to check for the presence of some novel metabolites which may be of significant use in controlling cell proliferation and thus drug development against cancer.

This is the first study describing the phytochemical content and anti-cell proliferative activity in *I. barbata* as this plant has never been reported earlier. The study of plant metabolites and presence of anti-cell proliferative activity in the bark and roots of *I. javanica* has also not yet been reported.

5. MATERIALS AND METHODS

5.1. Collection and authentication of plants and preparation of the plant extracts

The fresh and healthy leaves, flowers, bark, and roots of the experimental plants *I. pavetta*, *I. barbata*, *I. javanica* and *I. undulata* were collected from the campus of Acharya Jagadish Chandra Bose Indian Botanical Garden, Botanical Survey of India, Central National Herbarium, Kolkata, India. The plants were taxonomically authenticated with Blatter herbarium at the Centre itself. Leaves and bark of all above-mentioned four species of *Ixora* were collected for study, however additionally, flowers and roots of *I. barbata* and *I. javanica* were also collected for this study. Selected parts of all experimental plants were rinsed thoroughly with water and shade dried. Each of these were separately ground into a coarse powder using a mechanical grinder and stored in clean ambered colored glass bottles at room temperature for further analysis.

5.2. Procuration of Standard compounds and chemicals

The standard flavonoids rutin, quercetin, kaempferol were procured from Sigma & Merck Company and camptothecin from Sigma & Aldrich Company. All HPLC grade solvents were used for extraction purposes.

5.3. Extraction of dried powdered samples of leaf and flower by successive solvent Soxhlet method

Extraction of phytochemicals from leaf and flower samples carried out using successive solvent Soxhlet method [30]. The extraction was carried out using 25 g each of the dried leaf and flower powder using 250 ml each of three different HPLC grade solvents namely Petroleum ether (100%), Methanol (85%) and Ethanol (90%) in successive pattern. The solvent extracts obtained in each step were filtered using Whatman's filter paper no. 1 and the filtrates were concentrated in a rotary evaporator to obtain a semisolid mass. Thus, leaves and flowers extracted finally in ethanol using the above extraction method were used to detect anti cell proliferative activity on selected cancer cell lines and for estimation of total flavonoid content.

5.4. Extraction of dried bark and root powdered samples by Shaker extraction method

The extraction was carried out using the dried powdered samples of bark of all four species of *Ixora* and roots of *I. barbata* and *I. javanica* through the Shaker extraction process using 60% ethanol as a solvent. One gram of dried powder of the bark or root sample of respective plant species was each extracted in 10 ml of 60% ethanol for 8 hours in a shaker. Extracts were then passed through Whatman's filter paper No.1 and the filtrates centrifuged at 10,000 rpm for 15 mins [31]. The supernatants were concentrated in a crucible using a water bath to obtain a semi solid mass [15]. The semi solid mass of ethanolic bark and root extracts thus obtained from selected species of *Ixora* were used to study anti cell proliferative activity on three selected cancer cell lines and for estimation of total alkaloid content.

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5.5. Estimation of Total alkaloid content

Estimation of total alkaloid content in ethanolic bark extracts of *I. pavetta, I. javanica, I. barbata* and *I. undulata* and ethanolic root extracts of *I. javanica and I. barbata* were carried out by Bromocresol green (BCG) & Phosphate buffer test [32,33].

5.6. Estimation of Total flavonoid content

Estimation of total flavonoid content in ethanolic leaf extracts of all *Ixora* species under study and ethanolic flower extracts of *I. barbata and I. javanica* were carried out by aluminum chloride colorimetric assay [33].

5.7. Sulforhodamine B colorimetric assay for cytotoxicity screening (SRB assay)

Leaves and barks of all selected *Ixora species* and flowers and roots of only *I. barbata* and *I. javanica* extracted by methods listed above were studied for their anticancer activity against 3 cancer cell lines namely human lung cancer cell Line A-549 (A-549), human breast cancer cell line MDA-MB-231 (MDA-MB-231) and human leukemia cell line K-562 (K-562) using the SRB assay [34]. Cells were cultured as per NCI guidelines in appropriate U.S. origin heat inactivated media (RPMI 1640 GIPCO BRL media catalogue no.: 23400 supplemented with 10% FBS catalogue no.: RM10409 and 10% Antibiotic cocktail mixture of Gentamycin 12.5% + streptomycin 5% (antibacterial)+ Forcan 12.5% (antifungal). The effect of three commercially available standard flavonoids (rutin, quercetin, kaempferol) and one standard alkaloid (camptothecin) on the above selected cell lines was also analyzed simultaneously by the same method. These were labelled as Std 1, Std 2, Std 3 and Std 4 which were rutin (Std 1), quercetin (Std 2), kaempferol (Std 3), and camptothecin (Std 4) as denoted in the results. These standard drugs have been reported to have anticancer activity against other cell lines studied by some researchers [25,27,35-42]. The method followed is explained briefly- The cell lines under study were cultivated in medium mentioned above containing 2 mM L-glutamine and 10% fetal bovine serum. For the present study, 5,000 cells/well were inoculated into Corning incorporated 96 well microtiter plates (made up of Coster catalogue no. 3599). Assay was performed in a total volume of 100 µL as per NCI guidelines [43]. The microtiter plates were then incubated for 24 hrs. at 37°C under 95% air, 5% CO_2 and 100% relative humidity (Bio Safety Cabinet: Class II, Esco Micro; Model No. AC2-4EI, Sr No. 2008-33561). This was followed by the addition of experimental plant samples and standard drugs to be evaluated. Experimental drugs (semi-solid *Ixora* plant extracts and four standard drug sample powders) were solubilized in appropriate solvent (DMSO) at 100 mg/ml concentration and diluted to 1 mg/ml using milli-Q sterile/autoclaved water and stored frozen prior to use. Negative control contained the same % of DMSO as that of the compounds. During experimentation these were thawed and further diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml using complete medium already containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium. A final drug concentration of 10 µg/ml, 20 µg/ml, 40 µg/ml, and 80 µg/ml was thus obtained. Adriamycin (positive control) was also used in the same concentrations i.e., 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. Plates were incubated under standard conditions for 48 hrs. Assay was terminated using chilled trichloroacetic acid (TCA). SRB is an optimized assay for cytotoxicity screening of compounds for adherent cell lines like the A-549 and MDA-MB-231 and so 10% TCA was used for precipitation. For the K562 cell lines which grow as suspension culture, a higher concentration of TCA was used for precipitation. Cells were further fixed in situ by adding 50 µl of cold 30% (w/v) TCA followed by incubation at 4°C for one hour. The supernatant was discarded, and microtiter plates were washed five times with tap water. Air drying the washed plates is followed by addition of 50 µl of 0.4 % (w/v) SRB solution prepared in 1 % acetic acid and 20 minutes incubation at room temperature. Unbound dye is then removed by washing plates with 1 % acetic acid followed again by air drying. The bound stain is then eluted using a 10 mM Trizma base. Absorbance for the same is read at 540 nm with 690 nm reference wavelength in a 96 well Plate Reader (BioTek, Model: ELx808, Sr no 181019A) [43-45]. LC_{50}, GI_{50} and TGI data values represented the cytotoxicity of the experimental drugs (plant extracts and standard flavonoids and alkaloid) on each cell line. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Control wells contain only medium and do not contain any drug.

$$\text{Percent growth} = \frac{\text{Average absorbance of the test well}}{\text{Average absorbance of the control well}} \text{x } 100$$

Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the

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drug concentration levels. Percentage growth inhibition was calculated as: [Ti / C] x 100%. GI50 = Growth Inhibition of 50%, i.e., drug concentration results in a 50% reduction in the net protein increase indicating 50% inhibition of cell growth. GI50 is calculated from [(Ti - Tz) / (C - Tz)] x 100 = GI50, Tz = absorbance measurements at time zero. Ti = test growth in the presence of a drug at different concentration levels. Cells were observed under 20 X magnification using Inverted Microscope and photographed using Nikon, Model: Ecliple Ti-s, Sr No. 533505.

5.8. Statistical analysis

Statistical analysis software or IC-50 calculation software (Sigma plot, SPSS or microWin, Microtek was used for cell line analysis.

5.9. Ethical approvals

This study does not involve experiments on animals or human subjects.

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